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F Plasmid Conjugative DNA Transfer

THE TraI HELICASE ACTIVITY IS ESSENTIAL FOR DNA STRAND TRANSFER*

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The product of the *Escherichia coli* F plasmid *traI* gene is required for DNA transfer via bacterial conjugation. This bifunctional protein catalyzes the unwinding of duplex DNA and is a sequence-specific DNA transesterase. The latter activity provides the site- and strand-specific nick required to initiate DNA transfer. To address the role of the TraI helicase activity in conjugative DNA transfer *traI* mutants were constructed and their function in DNA transfer was evaluated using genetic and biochemical methods. A *traI* deletion/insertion mutant was transfer-defective as expected. A *traI* C-terminal deletion that removed the helicase-associated motifs was also transfer-defective despite the fact that the region of *traI* encoding the transesterase activity was intact. Biochemical studies demonstrated that the N-terminal domain was sufficient to catalyze *oriT*-dependent transesterase activity. Thus, a functional transesterase was not sufficient to support DNA transfer. Finally, a point mutant, TraI-K998M, that lacked detectable helicase activity was characterized. This protein catalyzed *oriT*-dependent transesterase activity *in vitro* and *in vivo* but failed to complement a *traI* deletion strain in conjugative DNA transfer assays. Thus, both the transesterase and helicase activities of TraI are essential for DNA strand transfer.

Conjugative DNA transfer (CDT)[1] provides an important means for the horizontal transfer of genetic traits among bacterial populations as well as the transphylic transfer of genes from *Agrobacterium tumifaciens* to plants. The latter process occurs by essentially the same mechanism. CDT has been extensively studied in a variety of conjugative plasmids and transposons, and details surrounding the biochemistry and regulation of this process are emerging (for reviews see Refs. 1–4). Conjugation begins with the formation of a stable mating pair between a donor cell and a recipient cell. In response to a "mating signal" that remains to be defined, DNA strand transfer is initiated from a site- and strand-specific nick within the plasmid *oriT* (origin of transfer). A single strand of DNA is then passed into the recipient cell with an overall 5′ to 3′ polarity and is stabilized in the recipient by one of two mechanisms (i) circularization and second strand synthesis or (ii) recombination into the recipient genome.

Molecular details surrounding the initiation of strand transfer are coming to light in several systems including the *Escherichia coli* F plasmid. In the F plasmid system a specific nucleoprotein complex, so far known to consist of the F-encoded proteins TraY and TraI and the host encoded integration host factor (5, 6), is required to initiate the physical transfer of single-stranded DNA (ssDNA). These components have been shown to be indispensable for transfer *in vivo* (7–13) and are sufficient to promote formation of a TraI-catalyzed site- and strand-specific nick on the transferred strand *in vitro* (5). The 192-kDa product of the F plasmid *traI* gene, known as DNA helicase I or TraI (14), catalyzes a site- and strand-specific transesterase reaction (15–17) and a helicase reaction (18–20). TraY and integration host factor are both site-specific DNA-binding proteins (21, 22) that bind *oriT* and apparently act to impose a particular architecture on that region of the plasmid to facilitate the binding of TraI and formation of the active initiation complex.

TraI is a key player in the initiation of CDT, and two distinct biochemical activities intrinsic to TraI are believed to be relevant to its biological role. One, a DNA transesterase activity, is characterized by the ability of the protein to introduce a sequence- and strand-specific nick at the F plasmid *oriT* (15, 16, 23, 24). The scissile phosphodiester bond is referred to as *nic*. The reaction involves a reversible transesterification between the 5′ phosphate of a guanosyl residue on the 3′ side of *nic* (G^{140}) (2), and a tyrosine near the N-terminal end of the protein.[2] TraI is also a nucleoside 5′-triphosphate hydrolysis-dependent 5′ to 3′ DNA helicase (18–20). The helicase-associated motifs of TraI are clustered in the C-terminal region of the protein. Both regions of TraI have similarities in their primary structure to other transesterases and helicases (2, 25–30).

The transesterase activity makes TraI indispensable for DNA transfer because formation of the sequence- and strand-specific nick is a required first step in strand transfer and is a plasmid-specific function. Interestingly, the overwhelming majority of CDT initiator transesterases do not contain identifiable helicase motifs nor do they exhibit helicase activity (for review see Ref. 24). However, TraI is believed to use its helicase activity to catalyze F plasmid unwinding concomitant with DNA transfer, thus providing the force required to drive a single strand of DNA from the donor to the recipient bacterium (14). This notion is based on the following lines of reasoning: (i) to transfer only one strand of the duplex plasmid unwinding is a mechanistic requirement, (ii) TraI physically interacts with the transferred (T)-strand and is required for transfer, and (iii) the 5′ to 3′ polarity of the TraI helicase activity is consistent

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[1] The abbreviations used are: CDT, conjugative DNA transfer; bp, base pair(s); ssDNA, single-stranded DNA; PCR, polymerase chain reaction.

[2] D. R. N. Byrd and S. W. Matson, unpublished results.

TABLE I
Bacterial strains and plasmids

Strain designation	Significant feature(s)	Source or reference
Cell strains		
HB101	leuB6, Δ(gpt-proA)62, lacY1, supE44, galK2, *recA13, rpsL20, xyl-5, mtl-1, Δ(mcrC-mrr), ara-14*	Ref. 46
JC7623	*thr-1, ara-14, leuB6, DE(gpt-proA)62, lacY1, sbcC201, tsx-33, gsr-0, glnV44(AS), galK2, LAM-, rac-0, sbcB15, hisG4(Oc), rfbD1, recB21, recC22, rpsL31, kdgK51, xylA5, mtl-1, argE3(Oc), thi-1*	Ref. 47
JC7623 derivatives		
DB4	pOX38T	This work
DB5	pOX38TΔtraI::kan	This work
DB6	pOX38TtraIΔC	This work
HMS174(λDE3)	F$^-$ *recA1 hsdR* ($r_{K12}^-m_{K12}^+$) Rif^R (DE3)	Novagen
HMS174(λDE3) derivatives		
DB10	pOX38T	This work
DB11	pOX38TΔtraI::kan	This work
DB20	pOX38TtraIΔC	This work
Plasmids		
pOX38T	Transfer F $^+$derivative	Ref. 39
pOX38TΔtraI::kan	Transfer F $^-$derivative	This work
pOX38TtraIΔC	F derivative with the C-terminal end of *traI* gene deleted	This work
Litmus 28	Cloning vector	New England Biolabs
pET11d	Expression vector	Novagen
pBSoriT	Bluescript plasmid with F *oriT*	Ref. 15
pBS	Bluescript plasmid lacking F *oriT*	Ref. 15
pACYCoriT	pLysE derivative with F *oriT*	This work
pET11d-traI	Expression vector with *traI* gene	This work
pET11d-traIN956	Expression vector with C-terminal deletion of *traI* gene	This work
pET11d-traIK998M	Expression vector with *traI* point mutant	This work

with the observed polarity of strand transfer (31, 32). In plasmid systems harboring a CDT initiator transesterase without helicase activity, this role is presumably filled via the recruitment of either a host- or plasmid-encoded helicase. Alternatively, strand displacement synthesis catalyzed by a DNA polymerase concomitant with DNA transfer could provide the ssDNA that is transferred to the recipient. Thus, there is neither precedent nor direct evidence demonstrating a requirement for the helicase activity of TraI in CDT.

To investigate the role of the TraI helicase activity in CDT, we made several *traI* mutants and evaluated their function in DNA transfer using genetic and biochemical methods. A *traI* deletion/insertion strain was transfer-defective as expected. A *traI* C-terminal deletion that removed the helicase-associated motifs was also transfer-defective despite the fact that the region of *traI* encoding the transesterase activity was intact. Biochemical studies demonstrated that the N-terminal 954 residues of TraI were sufficient to catalyze *oriT*-dependent transesterase activity *in vitro*, comparable with that of native TraI. Thus, a functional transesterase was not sufficient to support CDT. Finally, a point mutant, TraI-K998M, that lacked detectable helicase activity and had only minimal DNA-dependent ATPase activity was characterized. This protein catalyzed *oriT*-dependent transesterase activity *in vitro* and *in vivo* but failed to complement a *traI* deletion strain in conjugative DNA transfer assays.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Nucleic Acids—E. coli strains and plasmids used in this study are listed in Table I. DNA oligonucleotides are listed in Table II. Gene disruptions were constructed in JC7623 as described (33) and moved to other strains by transformation. Overexpression of the *traI* alleles cloned into pET11d (Novagen, Madison, WI) was in HMS174(DE3). Donor strains for mating experiments were constructed by transforming HMS174(DE3) with the appropriate pOX38T derivative and, where indicated, the appropriate pET11d-*traI* allele. Either JS4 or HB101 served as the recipient strain in all cases. Nucleic acids were quantified by spectroscopy at 260 nm.

Standard Genetic Techniques—Bacteria were grown in 2XYT or LB (34). LB was supplemented with 1.5% agar for plates. Medium was supplemented with antibiotics, as appropriate, at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 12 μg/ml; streptomycin, 30 μg/ml; kanamycin, 30 μg/ml; and chloramphenicol, 20 μg/ml. The liquid mating assay protocol was as follows. Donor and recipient strains were diluted 1:50 into LB from saturated overnight cultures grown under selection and allowed to grow to mid or late log phase in the absence of selection at 37 °C. Donors and recipients were then mixed at a volume ratio of one donor to nine recipients and incubated at 37 °C. After 5 min, the mating mixtures were diluted 1:10 into LB and incubated at 37 °C for an additional 30 min. The mating mixtures were then vigorously vortexed to disrupt mating pairs and 10-fold serial dilutions were prepared at room temperature in phosphate buffered saline. Appropriate dilutions were plated onto LB agar containing streptomycin and tetracycline to counter-select donors and unmated recipients while selecting for transconjugants. Aliquots of the unmated donor and recipient cultures were subjected to 10-fold serial dilution into phosphate buffered saline and plated onto LB agar containing the appropriate antibiotics to determine the viable donor cell count and the viable recipient cell count. Mating frequency was calculated as the number of transconjugants/100 viable donor cells.

Plasmid Constructions—The *traI* gene was amplified in a polymerase chain reaction (PCR) with Vent DNA polymerase using primers 049 and 050 (gift of Dr. Michael T. Howard, University of Utah) and pMP8 (35) as the template. The primers contain *Bam*HI sites that were used to clone the PCR product into the unique *Bam*HI site of the Litmus28 phagemid (New England Biolabs, Beverly, MA). This plasmid was designated L28traI. The traI gene was sequenced to ensure the absence of PCR-generated mutations.

L28traI was modified to replace a portion of the *traI* gene with a kanamycin resistance gene. The central portion of the *traI* coding region was removed by digestion with *Sph*I and *Bsr*GI, and the remainder of the plasmid (having 496 bp of 5′ and 938 bp of 3′ *traI* sequence) was gel purified. The kanamycin resistance gene (*kan*^{R}) from pACYC177 was cloned into a hybrid polylinker derived from pLit28 and pLit38. This allowed for the generation of a DNA fragment containing *kan*^{R} and having overhangs compatible with *Sph*I and *Bsr*GI. The appropriate DNA fragment was gel purified and ligated to the *traI* remnant to produce a plasmid in which the direction of transcription of the *kan*^{R} gene was the same as that of *traI*. A representative clone was selected on LB plates containing kanamycin, characterized by restriction mapping, and designated ptraIK.

The ptraIK construct was used to disrupt the *traI* locus on the F derivative pOX38T (the "T" denotes tetracycline resistance) (gift of Dr. Elisebeth Raleigh, New England Biolabs) by the following procedure. First, pOX38T was introduced into JC7623 by transformation. Several colonies were selected and screened for a transfer proficient phenotype, and a representative isolate was designated DB4 (Table I). A *Bam*HI fragment of ptraIK containing the disrupted *traI* gene was transformed

TABLE II
DNA oligonucleotides

Name	Sequence (5′ to 3′)	Description
db049	CGGAATTCGGATCCGTCAAAGGGATATACGTTTA	5′ PCR primer for *traI* gene
db050	CGAAGCTTGGATCCTATCAGTCTCCACCCAGGGT	3′ PCR primer for amplifying *traI* gene
db054	GCGGGTACCACCGCCTCCTTACCTTCGAGAATATGGC	Changes codon 955 from ATG (M) to GTA (V)
db055	GCGGGTACCGCTGATGGAGAGAGTACCTGGCG	Changes codon 955 from ATG (M) to GTA (V)
K998M	TGACCACACAGTTCAGGCCTGTGATGT	Changes codon 998 from AAG (K) to ATG (M)
K998WT	ACCCACACCGGCATAGCCCTGTAC	Mutagenesis primer for amplifying *traI* gene

into DB4. Transformants harboring the disrupted *traI* gene were selected on LB agar containing kanamycin and tetracycline. Deletion/insertion at the *traI* locus was confirmed by restriction mapping and Southern blot analysis of the pOX38T derivative (data not shown). The new pOX38T derivative was designated pOX38TΔtraI::kan, and the JC7623 derivative strain that harbored it was designated DB5.

The intact *traI* gene was initially subcloned from L28traI into pET11d using the *Bam*HI sites resident on each plasmid. This resulted in two ribosome binding sites upstream of *traI,* one site from pET11d and the native *traI* ribosome binding site. The pET11d ribosome binding site was deleted using the flanking *Xba*I and *Nhe*I sites. This construct was designated pET11d-traI.

A unique *Kpn*I/*Acc*651 site was introduced into the *traI* gene in pET11d-traI at codon 955. This was done by PCR with Vent DNA polymerase using pMP8 as the template, primers 049 and 054 to amplify the first 2865 bp of the gene, and primers 050 and 055 to amplify the last 2406 bp of the gene. The two PCR products were digested with *Kpn*I and ligated. As expected, ligation resulted in three products: a 5730-bp product and a 4812-bp product that were head to head ligations of the 5′ and 3′ halves of the gene, respectively, and the desired 5271-bp product. The latter was gel purified, digested with *Bam*HI, and cloned into the unique *Bam*HI site of pET11d. The *Kpn*I/*Acc*651 site was used to create a C-terminal deletion of the TraI protein. The construct described above was digested with *Acc*651 and *Bsr*GI, which leaves 2865 bp of 5′ and 938 bp of 3′ *traI* sequence. The ligation of these compatible cohesive ends brings a TAA stop codon into frame two codons after codon 955 and, therefore, eliminates expression of the C-terminal region (residues 955–1756), which includes the helicase-associated motifs. This construct was designated pET11d-traIN956.

To make the pOX38TtraIΔC deletion, the *Acc*651 and *Bsr*GI sites in L28traI were used to replace most of the *traI* C-terminal coding region, including the entire region encoding the helicase-associated motifs, with a kanamycin resistance marker. This plasmid was designated L28traIΔCK. The disrupted *traI* gene in L28traIΔCK was amplified with primers 049 and 050 and transformed into DB4. The *traI*ΔC disruption in pOX38T was assessed in the same manner as outlined above for pOX38TΔtraI. The plasmid was designated pOX38TtraIΔC, and the resulting derivative strain was designated DB6. pOX38T, pOX38TΔtraI, and pOX38TtraIΔC were introduced into HMS174(DE3) by transformation and the resulting strains were designated DB10, DB11, and DB20, respectively (Table I).

The plasmid pBSoriT was described previously (15). pACYCoriT is a derivative of pLysE (Novagen) containing the F plasmid *oriT* region. It was constructed by cloning the 932-bp *Pvu*II fragment from pBSoriT into pLysE cleaved with *Eco*RV. The construction was confirmed by restriction digest analysis.

pET11d-traIK998M is a derivative of pET11d-traI with a point mutation at codon 998 that changes the conserved lysine in helicase motif I to methionine. This mutation was made by PCR using primers K998M and K998WT (Table II) as described previously (36). The *traI*-K998M gene was sequenced to confirm the K998M mutation and to ensure the absence of other mutations.

TraIN956 Purification—One liter of HMS174(DE3) harboring pET11d-traIN956 was grown to an A_{600} of 0.7, and isopropyl-thio-B-D-galactopyranoside was added to a final concentration of 0.5 mM to induce protein expression. Following a 3-h incubation at 37 °C, ~8 g of cells were recovered by centrifugation and stored at −80 °C. The cell paste was suspended in 32 ml of cold lysis buffer (40 mM Tris-HCl, pH 8.3, 7.5% sucrose, 150 mM NaCl, 4 mM EDTA, 5 mM β-mercaptoethanol, 10 mM phenylmethane-sulfonyl fluoride). Lysozyme was added to 0.3 mg/ml, Brij-35 was added to 0.4%, and the preparation was stirred at 4 °C for 1 h. The lysate was sonicated to reduce viscosity and centrifuged for 60 min at 27,000 × *g*. Solid ammonium sulfate was added to the clarified supernatant to 60% saturation, and the resulting precipitate was recovered by centrifugation for 60 min at 27,000 × *g*. The pellet was suspended in 5 ml of Buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 20% glycerol) and dialyzed against 200 volumes of Buffer B containing 200 mM NaCl. This was designated Fraction II.

Fraction II was applied to a 10-ml heparin-agarose (Sigma) column (cross-sectional area, 0.79 cm^2) equilibrated in Buffer B + 0.2 M NaCl, and the column was washed to baseline as judged by Bradford protein assay. A 10 column volume NaCl gradient from 0.2 to 0.8 M in Buffer B was used to develop the column. The elution of TraIN956 was followed by Western blot using antibodies raised against purified TraI. The protein eluted between 0.37 and 0.44 M NaCl. Peak fractions were pooled and designated Fraction III. Fraction III was assayed for *oriT*-dependent transesterase activity as described below. In addition to a pronounced *oriT*-specific transesterase activity, a second activity consistent with a type II topoisomerase was evident, irrespective of the presence of *oriT.* This activity could be mitigated with heat treatment (70 °C for 5 min) with no apparent reduction in the *oriT*-specific nicking activity. The contaminating activity could also be removed by further purification.

Fraction III was applied to a 10-ml phosphocellulose column (cross-sectional area, 0.79 cm^2). A 10-column volume NaCl gradient from 0.1 to 0.7 M was used to develop the column. TraIN956 eluted over a range from 0.2 to 0.45 M NaCl. The purity of these fractions was determined by polyacrylamide gel electrophoresis in the presence of SDS. Faster migrating species, evident by Coomassie staining, strongly cross-reacted with polyclonal antibody raised to TraI, suggesting that they were derived from proteolysis of the 956-amino acid species. The peak fractions were pooled, quantified by Bradford protein assay, and used to assess the *oriT*-specific nicking activity of the N-terminal 956 residues of TraI.

TraI-K998M Purification—TraI-K998M was purified using TraI purification procedure described previously (19) with minor modifications.

DNA Helicase Assays—A partial duplex helicase substrate was made essentially as described (37). Briefly, a 91-base oligonucleotide was annealed to its complementary sequence on purified M13mp6 ssDNA at a 1:1 molar ratio. The partial duplex DNA was 3′ end-labeled using the Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P]dCTP. After phenol/chloroform extraction, the substrate was further purified on a Biogel A5M column. The void volume fractions were pooled, ethanol precipitated, and suspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA to a final concentration of ~5 fmol/μl.

DNA unwinding reaction mixtures (typically 20 μl) contained 25 mM Tris-HCl, pH 7.5, 20 mM NaCl, 3 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10 fmol of DNA substrate, and 2 mM ATP. Reaction mixtures were assembled at room temperature, and the reaction was initiated by the addition of enzyme. Incubations were at 37 °C for 20 min. The reaction mixtures were quenched by the addition of EDTA to 25 mM and SDS to 1%. Reaction products were resolved from the substrate on an 8% (20:1 cross-linking) native polyacrylamide gel.

ATPase Assays—Reaction mixtures (20 μl) were identical with those used for helicase assays with the following exceptions: 15 pmol [α-^{32}P]ATP was added to each reaction mixture (final concentration of ATP, 2 mM) that contained 0.75 μg of M13mp6 ssDNA instead of the partial duplex helicase substrate. Reactions were assembled at room temperature and initiated by the addition of enzyme. Incubations were at 37 °C for 10 min. The reactions were quenched by the addition of EDTA to a final concentration of 85 mM. 4 μl was spotted onto a cellulose polyethyleneimine thin layer chromatography plate (J. T. Baker, Phillipsburg, NJ) and allowed to dry, and the plates were developed with a mobile phase consisting of 1.0 M formic acid and 0.8 M LiCl. The plates were allowed to dry, and the degree of ATP hydrolysis was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Duplex DNA Relaxation Assays—Assays were done in a manner slightly modified from those described previously (15). In addition to the protein (usually present at 50–60 nM), a typical reaction mixture (20 μl) contained 6 nM pBSoriT DNA (either supercoiled or covalently closed

circular), 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, and 15% glycerol. Reactions were assembled at room temperature and incubated at 37 °C for 20 min. Reactions were stopped by the addition of Proteinase K (Roche Molecular Biochemicals) and SDS to final concentrations of 1 mg/ml and 0.25%, respectively, and allowed to incubate at 37 °C for an additional 20 min. The products were resolved on 0.8% agarose gels in the absence (in the case of sc-pBSoriT), or presence (in the case of ccc-pBSoriT) of ethidium bromide, followed by staining and/or destaining, as required to visualize the results. For quantification of the transesterase reaction catalyzed by TraI and TraI-K998M, pBSoriT was digested with *Xba*I to linearize the plasmid, and known amounts of plasmid DNA were loaded on the agarose gel to produce a standard curve. The fluorescence of the nicked DNA species was compared with the fluorescence of the standard curve to determine the amount of DNA that was nicked using an Eagle Eye imaging system (Stratagene, La Jolla, CA).

DNA Oligonucleotide Cleavage/Exchange Assays—Reaction conditions were the same as for the duplex DNA relaxation assays except the substrates were ssDNA oligonucleotides either 22 or 30 nucleotides in length containing the *nic* site in F *oriT*. Cleavage assays typically contained 0.5 pmol of one or the other 5′ end-labeled substrate molecule. The exchange reaction contained 0.5 pmol of ^{32}P-labeled 30-mer, and 5.0 pmol of unlabeled 22-mer. The reactions were assembled at room temperature, initiated by the addition of enzyme, and incubated at 37 °C for 30 min. They were stopped by the addition of 0.25 volumes of a solution containing 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol. The reactions were placed in a boiling water bath for 2–3 min and then loaded onto a 16% denaturing polyacrylamide gel (20:1 cross-linking ratio). Electrophoresis was at a constant power of 50 watts until the xylene cyanol dye was at the midpoint of the gel. The gels were visualized using either x-ray film autoradiography or a PhosphorImager.

In Vivo Nicking Assays—Cell lysates were prepared and analyzed as described previously (38). The agarose gels were transferred to a nitrocellulose membrane by Southern blot (36). A 535-bp *Sal*I-*Spe*I fragment of pACYCoriT was 3′ end-labeled using the Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P]dCTP. The preparation was phenol/chloroform extracted and further purified on a Biogel A5M column. The void volume fractions were pooled, ethanol precipitated, and suspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. This was used to probe the blot to detect and quantify supercoiled and nicked DNA species.

RESULTS

Purified TraI protein catalyzes two distinct biochemical reactions, a site- and strand-specific transesterase reaction, and a 5′ to 3′ helicase reaction (15–20). The transesterase provides the site- and strand-specific nick required to initiate DNA strand transfer and is, therefore, required for DNA strand transfer. The helicase is believed to unwind the duplex plasmid and thereby provide the force required to transfer ssDNA from the donor to the recipient (14). However, direct evidence to indicate that the helicase activity of TraI is required for DNA strand transfer is lacking. Moreover, other transesterases (with very few exceptions) that initiate CDT do not catalyze a helicase reaction. To determine whether the helicase activity of TraI is required for its function in DNA transfer, we made several *traI* mutants and tested each mutant for function using *in vivo* and *in vitro* assays.

The role of the *traI* gene product in CDT was evaluated using the self-transmissible F plasmid derivative pOX38T (39). This mini-F plasmid encodes all the proteins required to sponsor its own conjugative transfer as well as a gene encoding resistance to tetracycline to aid in selecting cells that have received the plasmid by CDT. The plasmid was introduced into *E. coli* HMS174(DE3) by transformation to construct the donor DB10 (Table I). This donor strain transferred pOX38T at high frequency to a recipient strain in standard liquid mating experiments (Table III). Because the recipient strain inherits the transmissible plasmid, it immediately becomes a "donor" strain and can transfer the plasmid to another recipient lacking the plasmid. In this way the self-transmissible pOX38T can be rapidly transferred to all the cells in a bacterial population.

A central region of the *traI* gene on pOX38T was deleted and replaced with a gene encoding kanamycin resistance as described under "Experimental Procedures" to construct pOX38TΔtraI. This deletion/insertion was constructed using convenient restriction sites and was designed to eliminate expression of an active protein. Western blots failed to detect the expression of protein that reacted with anti-TraI antibodies (data not shown). This plasmid was introduced into *E. coli* HMS174(DE3) by transformation to construct the isogenic donor DB11 (Table I). The pOX38TΔtraI plasmid cannot sponsor its own transfer to a recipient strain (Table III), as expected, because it lacks the essential *traI* gene, which is known to be required for DNA transfer (7). In all experiments we failed to detect even a single transconjugant and, therefore, transfer frequency was estimated to be less than the limit of detection in the mating assay. It should be noted that the actual transfer frequency is likely to be much lower.

TABLE III
Transfer complementation

Liquid mating assays were performed as described under "Experimental Procedures." The data represent the average and standard deviation about the mean of at least three independent experiments. The values reported as $< x$ represent the sensitivity of the experiment. In each case no transconjugants were obtained, and, therefore, the transfer frequency is likely to be much lower than the value reported.

Strain	Relevant genotype	Transfer frequency
		transconjugants/100 donors
DB10	pOX38T	7.05 ± 0.5
DB11	pOX38TΔtraI	$<8 \times 10^{-6}$
	pOX38TΔtraI/pET11d-traI	1.41 ± 0.27
	pOX38TΔtraI/pET11d-traIN956	$<8 \times 10^{-6}$
	pOX38TΔtraI/pET11d-traIK998M	$<7 \times 10^{-4}$
DB20	pOX38TtraIΔC	$<8 \times 10^{-6}$

DNA transfer was restored to this strain by providing TraI protein from a second plasmid, pET11d-traI, that expressed the *traI* gene (Table III). Transfer frequency in the complemented donor DB11 was consistently lower than transfer frequency in the donor DB10. This is due to the fact that pOX38T is competent to sequentially mobilize itself throughout a bacterial population as explained above, whereas pOX38TΔtraI can only transfer out of the donor strain and into the first recipient because the recipient does not contain the complementing plasmid. Therefore, it is not surprising that DB10 transfers pOX38T at somewhat higher frequencies than DB11 containing a complementing plasmid.

To evaluate the role of the TraI helicase domain in CDT, a second derivative of pOX38T, pOX38TtraIΔC, lacking the C-terminal end of *traI* was constructed. All the helicase-associated motifs were removed in this deletion, whereas the transesterase domain was left intact. The deletion plasmid was introduced into *E. coli* HMS174(DE3) to construct the isogenic donor strain DB20 (Table I). As observed with the plasmid lacking the *traI* gene, this plasmid was also unable to support its own transfer to a recipient bacterium (Table III). This result suggests that the presence of the N-terminal transesterase domain is not sufficient to support CDT. Providing full-length TraI from a second plasmid restored the loss of strand transfer in DB20 (data not shown).

The genetic data shown above indicate that the presence of the N-terminal transesterase, in the absence of the helicase, is not sufficient for F plasmid-mediated CDT. To demonstrate that the N-terminal end of TraI was competent to catalyze the appropriate transesterase reaction in the absence of the helicase-associated motifs, the C-terminal end of the *traI* gene was deleted from pET11d-traI to produce pET11d-traIN956, which expressed the N-terminal portion of TraI. This protein is analogous to the TraI produced from pOX38TtraIΔC in the donor strain DB20. TraIN956 was purified as described under "Ex-

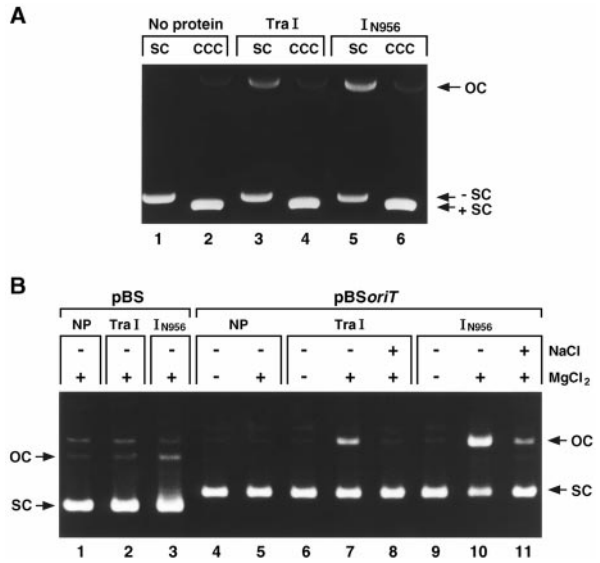

FIG. 1. **Comparison of the transesterase reaction catalyzed by TraI and TraIN956.** *A*, TraI and TraIN956 were incubated with negatively supercoiled (*sc*) pBSoriT DNA (*lanes 3* and *5*) or relaxed covalently closed circular (*ccc*) pBSoriT DNA (*lanes 4* and *6*) as described under "Experimental Procedures." *B*, each protein was incubated with supercoiled DNA (± *oriT* as indicated) in the presence (*lanes 2*, *3*, *7*, *8*, *10*, and *11*) or absence (*lanes 6* and *9*) of 6 mM $MgCl_2$. NaCl was added to the standard reaction to a final concentration of 150 mM (*lanes 8* and *11*). Reaction products were resolved on a 0.8% agarose gel, and the results were visualized by staining with ethidium bromide (0.3 μg/ml). *NP*, no protein; *OC*, open circular DNA.

perimental Procedures," and its biochemical activities were measured *in vitro*. TraIN956 exhibited no detectable helicase activity (data not shown), as expected, because all the helicase-associated motifs were deleted. However, TraIN956 was able to catalyze an *oriT*-dependent transesterase reaction (Fig. 1). In these assays, the ability of the protein to catalyze transesterification at the F plasmid *oriT* was measured by assessing conversion of a supercoiled plasmid containing *oriT* to a nicked species (15, 16). Both TraI and TraIN956 catalyzed a transesterification reaction (Fig. 1, *A*, *lanes 3* and *5*, and *B*, *lanes 7* and *10*). Previous studies (15) have shown that this reaction requires a negatively supercoiled DNA substrate; relaxed covalently closed circular DNA will not serve as a substrate. This was also true for TraIN956 (Fig. 1*A*, *lanes 4* and *6*).

The specificity of the transesterase reaction, its dependence on $MgCl_2$, and its inhibition by NaCl are demonstrated in Fig. 1*B*. Both TraI and TraIN956 failed to catalyze relaxation of a plasmid lacking the F plasmid *oriT* (Fig. 1*B*, *lanes 1–3*). The slight increase in open circular form DNA observed in *lane 3* is due to a minor topoisomerase contaminant (see "Experimental Procedures"). The presence of intermediate species between the supercoiled and open circular bands in *lane 3* is consistent with this interpretation. The transesterification reaction required $MgCl_2$ (Fig. 1*B*, *lanes 7* and *10*) and was inhibited by 150 mM NaCl (Fig. 1*B*, *lanes 8* and *11*) as previously reported (5). Thus, at this level of analysis, the transesterase reaction catalyzed by TraIN956 compared favorably with that catalyzed by the wild-type TraI.

Several site-specific DNA transesterases also catalyze cleavage of a ssDNA oligonucleotide whose sequence is derived from the cognate binding site of the protein. If a second oligonucleotide encompassing the same sequence, but of different length, is included in the reaction, then transesterase-catalyzed strand exchange can be observed (Fig. 2*A* and Refs. 17 and 40–43). Cleavage of a 5′ end-labeled 30-mer was observed using both TraI and TraIN956 and was dependent upon the presence of

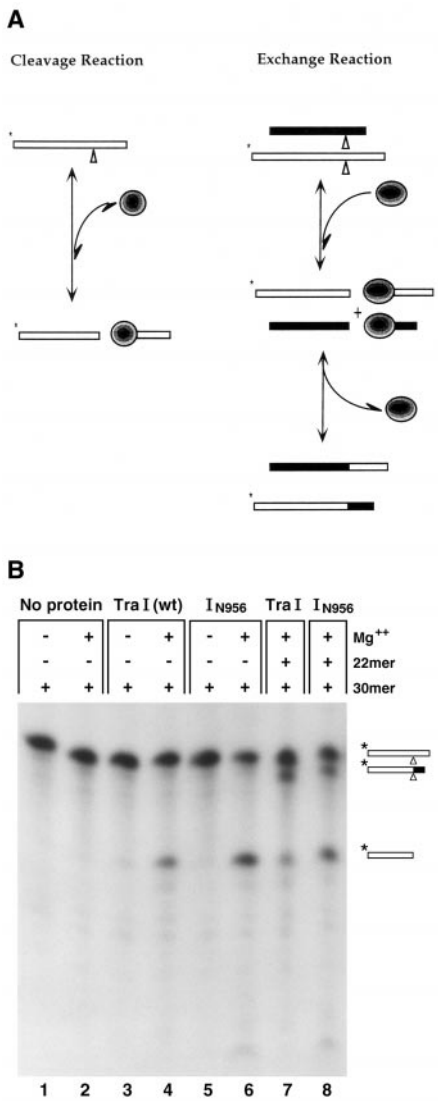

FIG. 2. **The cleavage and exchange reactions catalyzed by TraI and TraIN956.** *A*, a schematic representation of the oligonucleotide cleavage and exchange reactions, and the predicted products. *Asterisks* denote radioactive phosphates located on the 5′ end of DNA oligonucleotides. *Open* and *filled rectangles* represent 30-mer and 22-mer, respectively. *B*, autoradiograph of cleavage and exchange reactions. The expected reaction products are indicated at the *right*. Each protein was incubated with ^{32}P-labeled DNA (30-mer) in the presence (*lanes 4* and *6*) or absence (*lanes 3* and *5*) of 6 mM $MgCl_2$. Addition of a 10-fold molar excess of the unlabeled 22-mer resulted in the generation of novel 5′-end-labeled species with a predicted length of 28 bases (*lanes 7* and *8*). Reactions were as described under "Experimental Procedures." *wt*, wild type.

$MgCl_2$ (Fig. 2*B*, compare *lanes 3* and *4* with *lanes 5* and *6*). Strand exchange was observed upon addition of an unlabeled 22-mer to the reactions and was manifested by the generation of a labeled 28-mer (Fig. 2*B*, *lanes 7* and *8*). Both the cleavage and the exchange reactions catalyzed by TraIN956 were qualitatively equivalent to those catalyzed by wild-type TraI. Therefore, the N-terminal half of TraI protein expressed in the donor DB20 is a competent transesterase.

To rule out the possibility that TraI protein had a third, still undiscovered, activity in CDT that was compromised in the deletion mutant, a plasmid that expressed a TraI point mutant that eliminated helicase activity but retained transesterase activity was constructed. The plasmid pET11d-traIK998M expressed a TraI point mutant containing a lysine to methionine mutation in the essential lysine (Lys^{998}) in helicase motif I.

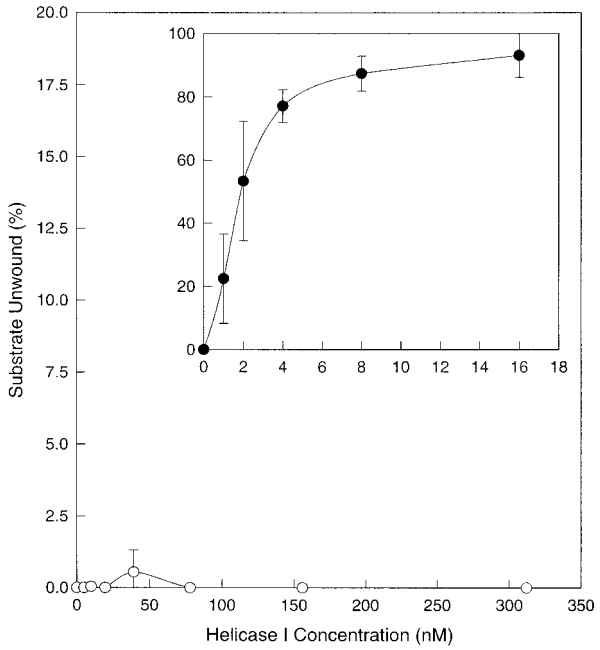

FIG. 3. **The helicase activity of TraI and TraI-K998M.** The helicase activity of TraI (*filled circles*) and TraI-K998M (*open circles*) was measured as described under "Experimental Procedures" using a 92-bp partial duplex substrate. The data represent the average of three independent experiments, and the error bars represent the S.D. about the mean. The fraction of the substrate unwound was calculated as described previously (45).

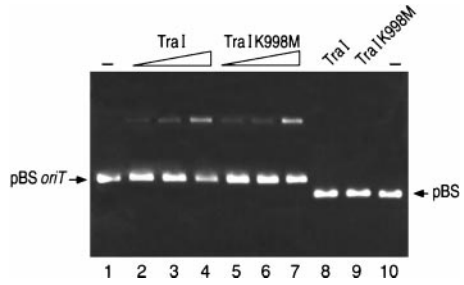

FIG. 4. **The transesterase activity of TraI and TraI-K998M.** The transesterase reaction catalyzed by TraI and TraI-K998M was measured as described under "Experimental Procedures." *Lane 1*, supercoiled pBSoriT DNA; *lanes 2–4*, supercoiled pBSoriT DNA and 2-fold increasing concentrations of purified TraI beginning at 38 nM; *lanes 5–7*, supercoiled pBSoriT DNA and 2-fold increasing concentrations of purified TraI-K998M beginning at 44 nM; *lane 8*, supercoiled pBS DNA and 112 nM purified TraI; *lane 9*, supercoiled pBS DNA and 123 nM purified TraI-K998M; *lane 10*, supercoiled pBS DNA and no protein. The 0.8% agarose gel was stained with EtBr (0.5 μg/ml).

This plasmid was introduced into the donor strain DB11 (containing pOX38TΔtraI), and DNA transfer was measured. This cell strain was transfer-deficient, indicating that the TraI point mutant cannot substitute for TraI (Table III).

To eliminate the unlikely possibility that this mutant protein lacked transesterase activity, the TraI-K998M mutant protein was purified to apparent homogeneity to evaluate its biochemical activities. The helicase, ssDNA-dependent ATPase, and transesterase activities of the mutant protein were measured and compared with the wild-type protein. The k_{cat} for ATP hydrolysis was reduced to less than 0.08% that of wild-type TraI (data not shown) as expected because the lysine residue is essential for the ATPase reaction catalyzed by helicases (44). The unwinding activity of TraI-K998M was measured using a 92-bp partial duplex substrate (Fig. 3). No detectable unwinding of this substrate by TraI-K998M was observed even at high protein concentration (312 nM). Wild-type TraI, by comparison, unwound nearly all of the partial duplex substrate at a concentration of 8 nM protein (Fig. 3, *inset*). The transesterase activity of TraI-K998M was measured using the agarose gel assay described above. TraI and TraI-K998M both catalyzed the conversion of supercoiled pBSoriT to an open circular, nicked form of the DNA (Fig. 4). Control experiments using a plasmid that lacked the F *oriT* sequence demonstrated that the reaction was specific for the F plasmid *oriT* (Fig. 4, *lanes 8–10*).

Although not likely, it was possible that TraI-K998M was not expressed in DB11 because of the presence of the point mutation. To ensure that expression of TraI and TraI-K998M were at equivalent levels, extracts were prepared from DB11 + pET11d-traI and DB11 + pED11d-TraI-K998M, and the level of TraI expression was determined by Western blot using antiserum directed against TraI. Both proteins were expressed in the donor strain at equivalent levels (data not shown). It should be noted that the level of TraI expression in these experiments was significantly higher than expression from the single copy of *traI* present on pOX38T because of the presence of the multicopy expression plasmid pET11d-traI. The higher level of expression of TraI did not have a negative impact on the complementation assays.

To confirm that TraI-K998M was active *in vivo*, the ability of the protein to function as a transesterase in the donor strain was assessed by directly measuring the fraction of relaxed F *oriT*-containing plasmid molecules in DB11 containing pET11d-traIK998M. For this purpose a small plasmid, pACYCoriT, containing the F *oriT* was transformed into DB11 harboring either pET11d-traI or pET11d-traIK998M. Conversion of supercoiled pACYCoriT to nicked, open circular pACYCoriT was analyzed on agarose gels and quantified (Fig. 5). The two alleles of *traI* were qualitatively comparable in their ability to nick pACYCoriT *in vivo*. It should be noted that conversion of 100% of the plasmid to a nicked form is not expected, even in the presence of high concentrations of TraI, because the nicked form of the plasmid is in equilibrium with the supercoiled form of the plasmid because of the reversible nature of the transesterification reaction catalyzed by TraI. Thus, TraI-K998M retains its ability to catalyze the transesterase reaction required to initiate strand transfer but fails to fulfill all the roles required of TraI in DNA transfer by virtue of its lack of helicase activity.

DISCUSSION

The experiments described above address the functional importance of the TraI helicase activity in the DNA transfer reaction associated with F plasmid-directed bacterial conjugation. Previous studies have shown that TraI catalyzes two distinct biochemical reactions, an F plasmid *oriT*-specific transesterase reaction and a 5′ to 3′ helicase reaction (15–20). The transesterase domain is located at the N-terminal end of the protein, and the helicase domain encompasses the remainder of the protein.[2] Because DNA strand transfer begins at the site- and strand-specific nick introduced within *oriT*, it is clear that the transesterase activity of TraI is essential for bacterial conjugation. However, the role of the helicase activity had not been directly tested. Indeed, most CDT initiator proteins do not manifest an intrinsic helicase activity (for review see Ref. 24). Therefore, it was of interest to determine whether the helicase activity associated with TraI was essential for CDT.

We have shown, using a deletion mutant lacking the C-terminal end of TraI and a point mutant that abolished the helicase activity of TraI, that the helicase activity of TraI is essential for CDT. Neither mutant was able to provide the activity necessary to sponsor DNA transfer or complement a strain harboring a deletion of the *traI* gene (Table III). Purifi-

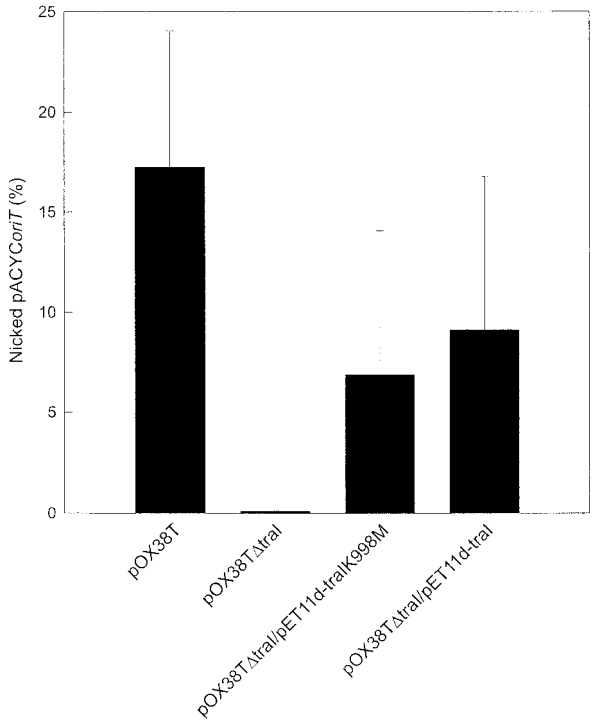

FIG. 5. **TraI and TraI-K998M catalyze a transesterification reaction *in vivo*.** The *in vivo* transesterase activity of TraI and TraI-K998M was measured as described under "Experimental Procedures." The fraction of pACYCoriT that was nicked in a cell strain lacking TraI was used as background. *First column*, pOX38T/pACYCoriT; *second column*, pOX38TΔtraI/pACYCoriT; *third column*, pOX38TΔtraI/pET-11d-TraIK998M/pACYCoriT; *fourth column*, pOX38TΔtraI/pET11d-traI/pACYCoriT. The data represent the averages of three independent experiments, and the error bars represent the S.D. about the mean.

cation and biochemical characterization of the proteins expressed in each case (TraIN956 andTraI-K998M) revealed that both proteins lacked helicase activity while retaining site- and strand-specific transesterase activity. Therefore, it was unlikely that the transesterification reaction was compromised *in vivo*. To ensure that this was the case, the ability of TraI-K998M to nick an F-*oriT* containing plasmid *in vivo* was demonstrated (Fig. 5). The fraction of the F *oriT*-containing plasmid that was nicked *in vivo* was essentially equivalent in the strain harboring the expression plasmid with wild-type *traI* and the strain containing the TraI-K998M point mutant. Because the plasmid is nicked but cannot be transferred, we conclude that the *oriT*-specific transesterase activity of TraI is not, by itself, sufficient to support DNA transfer. The helicase activity is also essential and is likely providing the force required to drive strand transfer from the donor to the recipient acting as a molecular motor. Thus, both activities associated with TraI are essential for its biological function in CDT.

A previous study (12) reached a different conclusion regarding the role of the helicase activity associated with TraI. These investigators suggested that deletion of the C-terminal end of TraI severely affected but did not abolish DNA transfer. This was interpreted as evidence for partial complementation by a host-encoded helicase. We were unable to detect any DNA transfer with the pOX38TtraIΔC deletion mutant used in this study. Assay conditions were varied considerably by titrating donor to recipient ratios, increasing the time allowed for mating and altering medium conditions (data not shown). Under no condition did we observe partial complementation of DNA transfer as measured in a mating assay. In addition, expression of the transesterase domain of the protein (TraIN956) in a strain lacking the *traI* gene failed to produce any partial complementation of DNA transfer (Table III). In this case the transesterase was overexpressed, and a significant fraction of the transmissible plasmid should have been nicked and competent for transfer. If strand transfer complemented by a host helicase were to occur as a low frequency event, it should have been detectable under these conditions. However, this was not the case, indicating that partial complementation by a host helicase does not occur. The reason for the discrepancy between the results reported here and previous results (12) is not clear. One possibility is the incorrect assignment of various deletions with specific polypeptides in the previous study, which was conducted prior to the complete mapping and sequencing of the distal end of the *tra* operon. It is now known that the transesterase active site is near the N-terminal end of TraI and a significant portion of the TraI sequence is required to produce an active helicase.[2] In contrast to the previous study, the mutants used here have been characterized at the nucleotide level. In the previous study it is likely that some low level of residual helicase activity was present in the complementation constructs that were active in DNA transfer. It should be noted that the helicase-associated motifs do not extend to the extreme C-terminal end of the *traI* gene. Therefore, it may be possible to delete a portion of the C-terminal end of the gene without compromising helicase activity.

The results presented here demonstrate that nicking at *oriT,* although necessary, is not sufficient for DNA transfer. The plasmid must also be unwound, and the helicase activity of TraI is essential for unwinding and subsequent DNA transfer. Thus, this bifunctional protein binds *oriT*, catalyzes a site- and strand-specific transesterification reaction and then unwinds the plasmid to produce the ssDNA that will be transferred to the recipient cell. Because unwinding is essential for CDT, other transmissible plasmids that lack a helicase associated with the CDT initiator protein must usurp a plasmid or cellular helicase for this purpose. The identity of this helicase is unknown in most cases. Alternatively, a DNA polymerase capable of strand displacement synthesis could simultaneously produce the ssDNA to be transferred to the recipient cell and synthesize the replacement strand on the donor plasmid. The mechanism used by conjugative plasmids lacking a CDT initiator protein with helicase activity remains to be discovered.

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REFERENCES

1. Ippen-Ihler, K., and Minkley, E., Jr. (1986) *Annu. Rev. Genetics* **20,** 593–624
2. Frost, L., Ippen-Ihler, K., and Skurray, R. (1994) *Microbiol. Rev.* **58,** 162–210
3. Lanka, E., and Wilkins, B. (1995) *Annu. Rev. Biochem.* **64,** 141–169
4. Firth, N., Ippen-Ihler, K., and Skurray, R. A. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) pp. 2377–2401, ASM Press, Washington, D.C.
5. Nelson, W., Howard, M., Sherman, J., and Matson, S. (1995) *J. Biol. Chem.* **270,** 28374–28380
6. Howard, M., Nelson, W., and Matson, S. (1995) *J. Biol. Chem.* **270,** 28381–28386
7. Willetts, N., and Maule, J. (1979) *Mol. Gen. Genet.* **169,** 325–336
8. Everett, R., and Willetts, N. (1980) *J. Mol. Biol.* **136,** 129–150
9. Manning, P., Kusecek, B., Morelli, G., Fisseau, C., and Achtman, M. (1982) *J. Bacteriol.* **150,** 76–88
10. Willetts, N., and Wilkins, B. (1984) *Microbiol. Rev.* **48,** 24–41
11. Traxler, B., and Minkley Jr., E. (1987) *J. Bacteriol.* **169,** 3251–3259
12. Traxler, B., and Minkley, E. J. (1988) *J. Mol. Biol.* **204,** 205–209
13. Gamas, P. Caro, L. Galas, D., and Chandler, M. (1987) *Mol. Gen. Genet.* **207,** 302–305
14. Abdel-Monem, M., Taucher-Scholz, G., and Klinkert, M. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 4659–4663
15. Matson, S., and Morton, B. (1991) *J. Biol. Chem.* **266,** 16232–16237
16. Reygers, U., Wessel, R., Muller, H., and Hoffman-Berling, H. (1991) *EMBO J.* **10,** 2689–2694
17. Sherman, J., and Matson, S. (1994) *J. Biol. Chem.* **269,** 26220–26226
18. Abdel-Monem, A., Durwald, H., and Hoffmann-Berling, H. (1976) *Eur. J. Biochem.* **65,** 441–449

19. Lahue, E., and Matson, S. (1988) *J. Biol. Chem.* **263,** 3208–3215
20. Dash, P Traxler, B., Panicker, M., Hackney, D., and Minkley, E., Jr. (1992) *Mol. Microbiol.* **6,** 1163–1172
21. Lahue, E., and Matson, S,. (1990) *J. Bacteriol.* **172,** 1385–1391
22. Tsai, M. M., Fu, Y.-H. F., and Deonier, R. C. (1990) *J. Bacteriol.* **172,** 4603–4609
23. Matson, S., Nelson, W., and Morton, B. (1993) *J. Bacteriol.* **175,** 2599–2606
24. Byrd, D., and Matson, S. (1997) *Mol. Microbiol.* **25,** 1011–1022
25. Gorbalenya, A., Koonin, E., Donchenko, A., and Blinov, V. (1989) *Nucleic Acids Res.* **17,** 4713–4729
26. Pansegrau, W., and Lanka, E. (1991) *Nucleic Acids Res.* **19,** 3455–3462
27. Ilyina, T., and Koonin, E. (1992) *Nucleic Acids Res.* **13,** 3279–3285
28. Koonin, E., and Ilyina, T. (1993) *Biosystems* **30,** 241–268
29. Pansegrau, W., Schroder, W., and Lanka, E. (1994) *J. Biol. Chem.* **269,** 2782–2789
30. Llosa, M., Bolland, S., and de la Cruz, F. *(1994) J. Mol. Biol.* **235,** 448–464
31. Ohki, M., and Tomizawa, J. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33,** 651–658
32. Rupp, W., and Ihler, G. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33,** 647–650
33. Jasin, M., and Schimmel, P. (1984) *J. Bacteriol.* **159,** 783–786
34. Sambrook, J. Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. A.1–A.3, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
35. Panicker, M. (1985) *The Role of the traD Gene Product in F Sex Factor-mediated Conjugation in Escherichia coli*. Ph.D. thesis, Carnegie-Mellon University, Pittsburgh, PA
36. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1994) *Current Protocols in Molecular Biology*, Vol. 1, pp. 8.5.5–8.5.7, John Wiley & Sons, Inc., New York
37. Matson, S. (1986) *J. Biol. Chem.* **261,** 10169–10175
38. Thompson, T. L., Centola, M. B., and Deonier, R. C. (1989) *J. Mol. Biol.* **207,** 505–512
39. Guyer, M., Reed, R., Steitz, J., and Low, K. (1980) *Cold Spring Harbor Symp. Quant. Biol.* **45,** 135–140
40. Hanai, R., and Wang, J. (1993) *J. Biol. Chem.* **268,** 23830–23836
41. Moscoso, M., Eritja, R., and Espinosa, M. (1997) *J. Mol. Biol.* **268,** 840–856
42. Pansegrau, W., Schroder, W., and Lanka, E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2925–2929
43. Scherzinger, E., Kruft, V., and Otto, S. (1993) *Eur. J. Biochem.* **217,** 929–938
44. Hall, M. C., and Matson, S. W. (1999) *Mol. Microbiol.* **34,** 867–877
45. Hall, M. C., Özsoy, A. Z., and Matson, S. W. (1998) *J. Mol. Biol.* **277,** 257–271
46. Boyer, H. W., and Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41,** 459–472
47. Kushner, S. R., Nagaishi, H., Templin, A., and Clark, A. J. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 824–827